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Species diversity of planktonic gastropods (Pteropoda and Heteropoda) from six ocean regions based on DNA barcode analysis

Robert M. Jennings [a,*], Ann Bucklin [a], Holger Ossenbrügger [b], Russell R. Hopcroft [c]

[a] Marine Sciences Department, University of Connecticut at Avery Point, 1080 Shennecossett Road, Groton, CT 06340, USA
[b] Leibniz-Institut für Meereswissenschaften, FB3: Marine Ecology, Düsternbrooker Weg 20, 24105 Kiel, Germany
[c] Institute of Marine Science, 120 O'Neill, P.O. Box 757220, University of Alaska Fairbanks, Fairbanks, AK 99775-7220, USA

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ABSTRACT

Pteropods and heteropods are two distinct groups of holoplanktonic gastropods whose species and genetic diversity remain poorly understood, despite their ubiquity in the world's oceans. Some species apparently attain near cosmopolitan distributions, implying long-distance dispersal or cryptic species assemblages. We present the first multi-regional and species-rich molecular dataset of holoplanktonic gastropods, comprising DNA barcodes from the mitochondrial cytochrome *c* oxidase I subunit gene (COI) from 115 individuals of 41 species sampled from six ocean regions across the globe. Molecular analysis and assessment of barcoding utility supported the validity of several morphological subspecies and forms (e.g. of *Creseis virgula* and *Limacina helicina*), while others were not supported (e.g. *Cavolinia uncinata*). Significant genetic variation was observed among conspecific specimens collected in different geographic regions for some species, particularly in euthecosomatous pteropods. Several species of euthecosomes showed no evidence of genetic separation among distant ocean regions. Overall, we suggest some taxonomic revision of the holoplanktonic gastropods will be required, pending a more complete molecular inventory of these groups.

1. Introduction

Although the accepted ancestral habitat of marine gastropods is the benthos, two large groups of species live entirely in the pelagic realm—the Pteropoda (Pelseneer, 1888) and Heteropoda (Children, 1842). Within each group, progressive adaptation to a holoplanktonic lifestyle has been hypothesized from fully shelled, to partially shelled, to unshelled forms (van der Spoel, 1976; van der Spoel and Dadon, 1999). Together comprising some 140 species, the two groups (herein referred to as holoplanktonic gastropods) can be numerically and functionally important components of planktonic food webs (Pane et al., 2004; Seibel and Dierssen, 2003). Apart from possessing a wing-shaped foot used in swimming ("ptero" means "wing"), several features of their physiology are suggested to be associated with a holoplanktonic lifestyle: adept planktonic predation facilitated by the sensitive eyes of heteropods, the swimming behaviors of some gymnosomes (unshelled pteropods) that aid their predation on thecosomes (fully shelled pteropods; Harbison and Gilmer, 1992; Lalli, 1970; Lalli and Gilmer, 1989), and the anti-predation compounds produced by some thecosomes, which reduce palatability (McClintock and Baker, 1998).

Holoplanktonic gastropods are found throughout the world's oceans. Many species are known from widely separated ocean basins, apparently attaining near-cosmopolitan distributions. For example, the unshelled heteropod *Pterotrachea coronata* is found between 40°N and 30°S in the Atlantic, and in similar latitudes of the Red Sea, Mediterranean Sea, Indian Ocean, and Pacific Ocean (van der Spoel, 1976). The gymnosome *Spongiobranchaea australis* has a circumantarctic distribution, and the gymnosome *Clione limacina* apparently has attained a broad bipolar distribution in latitudes poleward of 40°N and S, although the northern and southern populations are hypothesized to be distinct subspecies (van der Spoel, 1976; van der Spoel and Dadon, 1999).

Although the structure and anatomy of holoplanktonic gastropods have been well studied, few details are known about the life histories (e.g. population movement, reproduction, dispersal) or genetics of most species. It remains unknown whether species with such cosmopolitan distributions are genetically cohesive, or are made up of cryptic species assemblages. Furthermore, underscoring the wide array of morphological variation in shell and body forms, feeding structures, and foot morphology, taxonomists have proposed a finer division of regional subspecies and "formae" whose validity has never been genetically substantiated.

* Correspondence to: Biology Department, University of Massachusetts at Boston, 100 Morrissey Boulevard, Boston, MA 02125, USA. Tel.: +1 617 287 6615; fax: +1 617 287 6650.
E-mail address: rob.jennings@umb.edu (R.M. Jennings).

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The extraordinary diversity of holoplanktonic gastropod forms and life histories has long frustrated taxonomic classification at most levels (Tesch, 1946, 1949; van der Spoel, 1976). The monophyly of both pteropods and heteropods has been questioned in taxonomic literature, and the groups have been allied with various other gastropod lineages (see Colgan et al., 2007; Klussmann-Kolb and Dinapoli, 2006). The recent molecular phylogenetic analysis of Klussmann-Kolb and Dinapoli (2006) used portions of nuclear ribosomal DNA and the mitochondrial cytochrome oxidase *c* subunit I gene (COI), and recovered the monophyly of Pteropoda with high support, whereas most taxonomists still separate heteropods into three independent lineages (but see Colgan et al., 2007). To the extent that complex morphological features in benthic gastropod species are known to vary both geographically and ecologically (e.g. Irie and Morimoto, 2008; Minton et al., 2008), an independent analysis of holoplanktonic gastropod molecular variation and species diversity is needed.

Because pteropods and heteropods are both diverse and neglected, and because it can be difficult to accurately identify specimens by morphology alone, the groups form an appealing system for barcode analysis (e.g. Hebert et al., 2003). Patterns of DNA sequence variation of a portion of COI known as a DNA barcode facilitate identification of described species, reveal potential cryptic variation within them, and allow detection of new or undescribed species (Bucklin et al., 2010; Schindel and Miller, 2005; Stoeckle and Hebert, 2008; Wiens, 2007). Barcoding has been effective in revealing previously undetected patterns of genetic diversity in terrestrial systems (e.g. Clare et al., 2007; Hajibabaei et al., 2006; Pfenninger et al., 2007) and marine systems (e.g. crustaceans, Costa et al., 2007; chitons, Kelly et al., 2007; fish, Ward et al., 2005). The existence of non-overlapping distributions of genetic distance within vs. between species in the taxon of interest has been termed the "barcode gap" and is considered an important feature of operable barcoding systems (Meier et al., 2008; Meyer and Paulay, 2005); however, means and standard deviations of inter- vs. intraspecific distances appear to be more commonly reported in the barcoding literature.

To our knowledge, no study has combined broad taxonomic sampling of holoplanktonic gastropods, wide-ranging geographic sampling, and molecular analysis; however, barcodes have proven useful in the broader phylogenetic analysis of several gastropod lineages (Remigio and Hebert, 2003). Therefore, to better understand the diversity of, and potential for cryptic species in, holoplanktonic gastropods, we barcoded roughly 40 species obtained in association with several Census of Marine Life (CoML) field projects, including the Census of Marine Zooplankton (CMarZ), the Arctic Ocean Diversity project (ArcOD), and the Census of Antarctic Marine Life (CAML). These species represent a significant multi-regional and -species collection effort, involving eight major research cruises throughout the world's oceans, and they establish the first large barcode dataset for pteropods and heteropods—indeed, for the majority of the species herein, no sequences for any gene were publicly available previously.

2. Methods

2.1. Collection and Identification of Specimens

The gastropods analyzed in this study were collected on eight cruises (Fig. 1). Two cruises sampled waters near Australia: a cruise in January/February of 2007 in the Southern Ocean south of Australia on the *R/V Aurora Australis* (Aurora-Australis-2007.3), and a cruise in November/December 2006 in the Indian Ocean northwest of Australia on the *R/V Galathea* (Galathea-2006). Four cruises sampled high latitude waters in the Northern Hemisphere: the *R/V Alpha Helix* to Prince Williams Sound in December 2003 (Alpha-Helix-2003), and to the Gulf of Alaska in July 2004 (Alpha-Helix-2004), the USCG *Healy* to the Canada Basin in June/July 2005 (Healy 05/2), and the FS *Polarstern* in August-October 2007 to Arctic waters north of Europe (PS-ARK-23-2). Finally, two cruises sampled the Atlantic: a cruise to the Sargasso Sea (Northwest Atlantic) in April 2006 on the *R/V R.H. Brown* (RHB0603), and a cruise that transited the eastern Atlantic from the Cape Verde Islands to South Africa on the FS *Polarstern* in November/December 2007 (PS-ANT-24-1).

Fig. 1. Location of the cruises providing specimens in this study. Cruise symbols on this map are used to designate locations of specimens in the gene trees.

On each cruise, live gastropods were identified to species (Polarstern cruise: H. Ossenbrügger; all other cruises, R. Hopcroft) and preserved in 95% ethanol. In all cases, vouchers of at least one additional individual taken from the same net tow have been retained, or as necessary, a minimal amount of tissue of an individual specimen was excised for DNA and the remainder retained as the voucher. All vouchers are therefore paragenophores (*sensu* Pleijel et al., 2008). Photographs were taken of specimens before dissection when possible. In some cases, specimens were identified to species, but subspecies/forma could not be determined. Vouchers and images are housed in A. Bucklin's lab at the University of Connecticut and are accessible by request.

2.2. Molecular analysis

For some specimens, DNA extraction, PCR amplification, and sequencing took place during the cruise. Other specimens were transported to the University of Connecticut (UConn) for analysis. Procedures and equipment were the same for all specimens. Genomic DNA was extracted from up to 25 mm^3 of tissue per individual using the DNeasy kit (Qiagen, Valencia, CA). A 660 bp fragment of the COI gene was amplified using the primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) from Folmer et al. (1994). PCRs were performed in 50 μL volumes with the following reagents: 1 × GoTaq Flexi buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 2 pmol dNTPs, 1.2 pmol of each primer, approximately 50 ng extracted genomic DNA, and 1 U of Taq polymerase (Promega, Madison, WI). Reactions were cycled under the following protocol: initial denaturation, 95 °C for 5 min.; 35 cycles of 95 °C for 30 sec., 50 °C for 45 sec, 72 °C for 1 min; final extension, 72 °C for 5 min. PCRs were purified using the QIAquick PCR Purification Kit (Qiagen) and eluted in 10 mM Tris-HCL.

Sequencing reactions were performed for both strands using ABI BigDye Terminators v3.1 (ABI, Foster City, CA) in one-eighth reaction format, purified via ethanol precipitation, and electrophoresed on an ABI 3130 Automated DNA Sequencer.

Sequences were assembled in Sequencher (GeneCodes, Inc., Ann Arbor, MI) and manually edited, then exported into BioEdit (Hall, 2007)) to ensure that they coded for amino acids with no stop codons or frameshifts. To check for possible contamination, all sequences were compared with a database of all barcodes produced in the UConn laboratory, and were screened against the GenBank database using BLAST (Altschul et al., 1990). Once verified, the COI sequences were aligned as amino acids using the CLUSTAL algorithm (Larkin et al., 2007) in BioEdit. This alignment was checked for potential artifactual translated sequences, and manually edited for consistency and to remove primer regions. The final dataset contained 115 specimens from 41 species (not counting subspecies and formae separately) from the cruises described above (Table 1), which have been deposited in GenBank (Accession Numbers FJ876837-FJ876951, FJ602528 and FJ602529) and linked to appropriate vouchers. Twelve COI sequences for planktonic gastropods previously deposited in GenBank by others were downloaded and included in analyses for comparison (Table 2).

Determining the proper outgroups with which to root the heteropod and pteropod trees is nontrivial given the high number and diversity of relevant gastropod lineages (caenogastropods and opisthobranchs, respectively) and varied taxonomic affiliations suggested by morphologists. Colgan et al. (2007) consistently recovered the Pterotracheidae (unshelled heteropods) among 10 hypsogastropod families they labeled the "GC group". Therefore, our outgroup to heteropods was composed by obtaining from GenBank the COI sequences from two species of separate genera per "GC group" family, or from one species per family if only one was available. Although recent molecular phylogenetic work

Table 1
Collection information for specimens analyzed in this study.

Taxonomy & Species	Specimen Voucher	Latitude Longitude	Collection Date	Ocean Region	Cruise	GenBank Accession
HETEROPODS (Pterotracheoidea)						
Atlantidae						
Atlanta gaudichaudi	Ga30.1.1	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876837
Atlanta gaudichaudi	Ga30.1.2	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876838
Atlanta gaudichaudi	Ga30.1.3	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876839
Atlanta inclinata	Ga24.10.1	13.42 S 0.65 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876840
Atlanta inclinata	Ga24.8.2	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876841
Atlanta inclinata	Ga24.9.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876842
Atlanta inclinata	Ga67.1.1	11.68 N 20.42 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876847
Atlanta cf. inclinata	Ga24.4.2	25.06 N 60.62 W	21-Apr-2006	NW Atlantic	RHB0603	FJ876843
Atlanta cf. inclinata	Ga24.4.3	25.06 N 60.62 W	21-Apr-2006	NW Atlantic	RHB0603	FJ876844
Atlanta sp.	Ga24.5.1	25.06 N 60.62 W	21-Apr-2006	NW Atlantic	RHB0603	FJ876845
Atlanta peronii	Ga03.2.1	33.57 N 69.65 W	14-Apr-2006	NW Atlantic	RHB0603	FJ876846
Oxygyrus keraudrenii	Ga38.6.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876848
Oxygyrus keraudrenii	Ga38.6.2	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876849
Pterotracheidae						
Firoloida demarestia	Ga41.2.1	14 N 55 W	25-Apr-2006	NW Atlantic	RHB0603	FJ876850
Firoloida demarestia	Ga41.4.1	14 N 55 W	25-Apr-2006	NW Atlantic	RHB0603	FJ876851
Pterotrachea coronata	Ga50.4.2	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876852
Pterotrachea coronata	Ga50.6.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876853
Pterotrachea hippocampus	Ga20.1.2	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876854
Pterotrachea hippocampus	Ga20.2.1	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876855
PTEROPODS						
Euthecosomata						
Cavolinia gibbosa	Ga05.1.1	33.52 N 69.96 W	13-Apr-2006	NW Atlantic	RHB0603	FJ876856
Cavolinia globulosa	Ga43.3.1	14 N 55 W	25-Apr-2006	NW Atlantic	RHB0603	FJ876857
Cavolinia longirostris	Ga32.1.1	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876859
Cavolinia longirostris	Ga32.1.2	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876860
Cavolinia tridentata atlantica	Ga57.1.1	46.35 S 140.54 E	22-Jan-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876861
Cavolinia uncinata	Ga29.6.2	17.47 S 121.43 E	09-Nov-2006	SE Indian	Galathea_2006	FJ876864
Cavolinia uncinata	Ga29.7.1	16.23 S 119.62 E	15-Nov-2006	SE Indian	Galathea_2006	FJ876865
Cavolinia uncinata	Ga29.9.1	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876866
Cavolinia uncinata	Ga68.2.3	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876858
Cavolinia uncinata uncinata	Ga29.1.1	24.95 N 60.53 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876862
Cavolinia uncinata uncinata	Ga29.9.2	14 N 55 W	25-Apr-2006	NW Atlantic	RHB0603	FJ876863
Cavolinia uncinata uncinata uncinata	Ga82.3.1	13.42 S 0.65 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876867
Cavolinia uncinata uncinata uncinata	Ga82.3.2	13.42 S 0.65 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876868
Clio cuspidata	Ga59.1.1	50.00 S 149.42 E	08-Feb-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876869
Clio cuspidata	Ga59.1.3	50.00 S 149.42 E	08-Feb-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876870
Clio cuspidata	Ga59.2.1	13.16 S 0.32 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876871
Clio pyramidata	Ga01.4.1	16.02 S 119.32 E	17-Nov-2006	SE Indian	Galathea_2006	FJ876874
Clio pyramidata	Ga01.4.2	16.02 S 119.32 E	17-Nov-2006	SE Indian	Galathea_2006	FJ876875
Clio pyramidata antarctica	Ga60.1.1	50.00 S 149.42 E	08-Feb-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876876
Clio pyramidata lanceolata	Ga01.1.2	33.52 N 69.96 W	13-Apr-2006	NW Atlantic	RHB0603	FJ876872
Clio pyramidata lanceolata	Ga01.1.3	33.52 N 69.96 W	13-Apr-2006	NW Atlantic	RHB0603	FJ876873
Clio pyramidata lanceolata	Ga75.1.1	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876877
Clio pyramidata lanceolata	Ga75.2.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876878
Clio pyramidata lanceolata	Ga75.3.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876879
Clio recurva	Ga58.1.1	45.00 S 142.98 E	20-Jan-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876880
Clio recurva	Ga63.5.1	50.00 S 149.42 E	08-Feb-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876881

Table 1 (*continued*)

Taxonomy & Species	Specimen Voucher	Latitude Longitude	Collection Date	Ocean Region	Cruise	GenBank Accession
Clio recurva	Ga63.6.1	46.35 S 140.54 E	22-Jan-2007	Southern Ocean	Aurora_Australis_2007.3	FJ876882
Clio recurva	Ga63.1.1	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876883
Clio recurva	Ga63.2.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876884
Clio recurva	Ga63.3.1	13.16 S 0.32 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876885
Clio recurva	Ga63.4.1	25.6 S 9.74 E	21-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876886
Clio sp.	Ga87.1.1	25.6 S 9.74 E	21-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876887
Creseis acicula	Ga19.4.1	16.02 S 119.32 E	17-Nov-2006	SE Indian	Galathea_2006	FJ876888
Creseis virgula virgula	Ga53.1.1	19.76 N 54.61 W	23-Apr-2006	NW Atlantic	RHB0603	FJ876889
Creseis virgula virgula	Ga14.1.1	29.88 N 70.07 W	16-Apr-2006	NW Atlantic	RHB0603	FJ876890
Creseis virgula conica	Ga77.1.1	1 S 9.01 W	13-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876891
Creseis virgula conica	Ga77.2.2	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876892
Cuvierina columnella	Ga06.1.1	30 N 70.03 W	15-Apr-2006	NW Atlantic	RHB0603	FJ876893
Cuvierina columnella	Ga06.2.1	29.48 N 70.51 W	17-Apr-2006	NW Atlantic	RHB0603	FJ876894
Cuvierina columnella	Ga06.4.1	24.99 N 59.99 W	19-Apr-2006	NW Atlantic	RHB0603	FJ876895
Cuvierina columnella	Ga06.6.1	16.64 S 120.22 E	13-Nov-2006	SE Indian	Galathea_2006	FJ876896
Cuvierina columnella	Ga06.7.2	16.44 S 119.92 E	14-Nov-2006	SE Indian	Galathea_2006	FJ876897
Diacria major	Ga02.1.1	33.52 N 69.96 W	13-Apr-2006	NW Atlantic	RHB0603	FJ876908
Diacria major	Ga02.12.1	16.44 S 119.92 E	14-Nov-2006	SE Indian	Galathea_2006	FJ876911
Diacria major	Ga02.13.1	16.23 S 119.62 E	15-Nov-2006	SE Indian	Galathea_2006	FJ876912
Diacria major	Ga02.2.1	33.52 N 69.96 W	13-Apr-2006	NW Atlantic	RHB0603	FJ876914
Diacria quadridentata	Ga07.2.1	24.99 N 59.99 W	19-Apr-2006	NW Atlantic	RHB0603	FJ876901
Diacria quadridentata	Ga07.2.2	24.99 N 59.99 W	19-Apr-2006	NW Atlantic	RHB0603	FJ876902
Diacria rampali	Ga70.1.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876903
Diacria rampali	Ga70.1.2	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876904
Diacria rampali	Ga70.4.1	13.16 S 0.32 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876905
Diacria rampali	Ga70.4.2	13.16 S 0.32 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876906
Diacria rampali	Ga70.4.3	13.16 S 0.32 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876907
Diacria cf. rampali	Ga70.5.1	25.6 S 9.74 E	21-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876898
Diacria cf. rampali	Ga70.5.2	25.6 S 9.74 E	21-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876899
Diacria cf. rampali	Ga70.6.1	25.6 S 9.74 E	21-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876900
Diacria trispinosa	Ga02.11.1	25.6 S 9.74 E	21-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876909
Diacria trispinosa	Ga02.6.1	24.68 N 20.75 W	05-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876910
Diacria trispinosa	Ga02.13.2	16.23 S 119.62 E	15-Nov-2006	SE Indian	Galathea_2006	FJ876913
Diacria trispinosa	Ga02.8.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876915
Diacria trispinosa	Ga02.9.1	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876916
Diacria trispinosa	Ga02.9.2	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876917
Diacria trispinosa	Ga02.9.3	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876918
Hyalocylis striata	Ga09.2.1	24.99 N 59.99 W	19-Apr-2006	NW Atlantic	RHB0603	FJ876919
Hyalocylis striata	Ga09.4.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876920
Hyalocylis striata	Ga09.5.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876921
Hyalocylis striata	Ga09.6.2	13.42 S 0.65 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876922
Limacina helicina helicina	Ga56.1.1	60.54 N 147.80 W	03-Dec-2003	Prince Williams Sound	Alpha-Helix-2003	FJ876923
Limacina helicina helicina	Ga56.2.1	87.02 N 146.35 W	04-Sep-2007	Arctic	PS-ARK-23-2	FJ876924
Limacina helicoides	Ga62.1.1	11.68 N 20.42 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876925
Limacina helicoides	Ga62.2.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876926
Limacina inflata	Ga11.1.1	29.88 N 70.07 W	16-Apr-2006	NW Atlantic	RHB0603	FJ876927
Limacina inflata	Ga11.1.2	29.88 N 70.07 W	16-Apr-2006	NW Atlantic	RHB0603	FJ876928
Limacina inflata	Ga11.1.3	29.88 N 70.07 W	16-Apr-2006	NW Atlantic	RHB0603	FJ876929
Limacina inflata	Ga11.2.2	24.68 N 20.75 W	05-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876930
Limacina inflata	Ga11.3.1	24.68 N 20.75 W	05-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876931
Psuedothecosomata						
Corolla spectabilis	Ga27.3.2	16.44 S 119.92 E	14-Nov-2006	SE Indian	Galathea_2006	FJ876935
Cymbulia sibogae	Ga80.1.2	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876932
Gleba cordata	Ga27.1.1	25 N 59.95 W	19-Apr-2006	NW Atlantic	RHB0603	FJ876933
Gleba cordata	Ga27.2.1	24.99 N 59.99 W	19-Apr-2006	NW Atlantic	RHB0603	FJ876934
Peracle bispinosa	Ga31.4.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876936
Peracle bispinosa	Ga31.4.2	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876937
Peracle bispinosa	Ga31.4.3	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876938
Peracle bispinosa	Ga31.6.1	3.21 N 14.6 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876939
Peracle valdiviae	Ga86.1.1	13.42 S 0.65 W	17-Nov-2007	SE Atlantic	PS-ANT-24-1	FJ876940
Gymnosomata						
Clione limacina	Ga04.1.1	33.52 N 69.96 W	13-Apr-2006	NW Atlantic	RHB0603	FJ876941
Clione limacina	Ga04.2.1	58.10 N 147.79 W	28-Jun-2004	Gulf of Alaska	Alpha-Helix-2004	FJ876942
Clione limacina	Ga04.3.1	73.0 N 156.93 W	24-Jun-2005	Canadian Basin	Healy 05/2	FJ876943
Clione limacina	Ga04.4.1	74.58 N 151.94 W	11-Jul-2005	Canadian Basin	Healy 05/2	FJ876944
Pneumoderma violaceum	Ga66.1.1	11.38 N 20.35 W	08-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876945
Pneumodermopsis macrochira	Ga16.1.1	29.87 N 70.08 W	16-Apr-2006	NW Atlantic	RHB0603	FJ876946
Pneumodermopsis macrochira	Ga16.2.1	29.83 N 70.24 W	16-Apr-2006	NW Atlantic	RHB0603	FJ876947
Pneumodermopsis macrochira	Ga16.3.1	24.79 N 60.36 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876948
Schizobrachium polycotylum	Ga72.1.1	3.51 N 14.01 W	11-Nov-2007	NE Atlantic	PS-ANT-24-1	FJ876949
Thliptodon diaphanus	Ga28.1.1	24.83 N 60.45 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876950
Thliptodon diaphanus	Ga28.2.1	24.79 N 60.36 W	20-Apr-2006	NW Atlantic	RHB0603	FJ876951

Table 2
Sequence information for outgroup species obtained from GenBank.

Higher Taxonomic Group	Family	Species	Accession
Heteropoda	Pterotracheidae	*Pterotrachea coronata*	DQ916505
Pteropoda	Euthecosomata	*Cavolinia uncinata*	DQ237997
Pteropoda	Euthecosomata	*Clio pyramidata*	DQ238000
Pteropoda	Euthecosomata	*Creseis* sp.	DQ280021
Pteropoda	Euthecosomata	*Cuvierina columnella*	DQ237998
Pteropoda	Euthecosomata	*Diacria quadridentata*	DQ238001
Pteropoda	Euthecosomata	*Hyalocylis striata*	DQ237999
Pteropoda	Euthecosomata	*Limacina helicina antarctica*	AY227378
Pteropoda	Euthecosomata	*Limacina helicina helicina*	AY227379
Pteropoda	Gymnosomata	*Clione limacina*	AY227377
Pteropoda	Gymnosomata	*Pneumoderma violaceum* (*P. atlanticum*)	DQ238003
Pteropoda	Gymnosomata	*Spongiobranchaea australis*	DQ238002

(e.g. Grande et al., 2004a, 2004b) has attempted to reconstruct the phylogeny of Opisthobranchia (in which pteropods are usually placed), a pteropod representative is rarely included. Morphological taxonomy has generally allied the gymnosomatous pteropods to anaspideans (van der Spoel and Dadon, 1999), but the associations of other pteropod groups have been more varied and more tenuous. Moreover, the phylogenetic relationships of the higher taxa are themselves poorly understood. To be thorough, opisthobranch COI sequences were obtained from GenBank in a similar manner as for heteropods (two genera per family if possible, one genus per family if not). For both the heteropod and pteropod outgroups, sequences were removed which were too short or showed irregularities (e.g. strings of N's, lack of a perfect open reading frame, or many non-consensus amino acids). This methodology resulted in outgroups of 16 hypsogastropods for heteropods and 39 opisthobranchs for pteropods (Table 3).

To investigate levels of DNA sequence divergence, Kimura 2-parameter distances (K2P, Kimura, 1980) were computed separately for pteropods and heteropods, between all pairs of sequences in MEGA 4.0 (Tamura et al., 2007), with gaps excluded on a pairwise basis. The K2P model was selected to allow comparison to other barcoding work. Distances were summarized in histograms at four hierarchical taxonomic levels: a, within species; b, among species within each genus; c, among genera within each family; and d, among families within the pteropods and heteropods.

To investigate the evolutionary relationships among gastropod species, separate gene trees for pteropods and heteropods were inferred using maximum likelihood (ML) and Bayesian methods. For consistency between methods, the general time-reversible (GTR) model of DNA sequence evolution was chosen under Akaike's Information Criterion using MrModelTest version 2.3 (Nylander, 2004), with an estimated proportion of invariant sites (+I) and mutation rates for the remaining sites drawn from a gamma distribution (+G). To estimate the ML tree, the hill-climbing algorithm of Guindon and Gascuel (2003) was performed online via the PHYML web server (Guindon et al., 2005), using SPR+NNI tree improvement with 10 random starting trees, the chosen GTR+I+G model, and the initial starting tree made by neighbor joining. The single tree with highest likelihood was selected, with nodal support assessed using the approximate likelihood ratio test (aLRT, Anisimova and Gascuel, 2006) as implemented in PHYML. To estimate the Bayesian tree, BEAST v1.5.3 (Drummond and Rambaut, 2007) was used, employing the GTR+I+G model of sequence evolution, and the relaxed uncorrelated lognormal molecular clock model (Drummond et al., 2006) of rate heterogeneity among branches. A starting tree was built via UPGMA, and the Yule process tree prior was chosen. All other priors were set to default options, and tuning parameters were adjusted as per the BEAST v1.5.3 manual (section 2.6.2). BEAST was run for 50,000,000 generations, sampling every 5000 generations, and the logfile was then examined with TRACER (Rambaut and Drummond, 2007) to ensure adequate effective sample sizes (ESSs) for key parameters, and to determine

Table 3
Sequence information for outgroups used to root heteropod and pteropod trees. 3A, outgroup for heteropods; 3B, outgroup for pteropods. Bold entries in 3B indicate species used in the final outgroup for pteropods.

Higher Taxonomic Group	Family	Species	Accession
3A. **Heteropod Outgroup: hypsogastropods**			
Littorinimorpha	Anabathridae	*Pisinna albizona*	DQ916499
Littorinimorpha	Eatoniellidae	*Crassitoniella flammea*	DQ916498
Littorinimorpha	Hipponicidae	*Antisabia foliacea*	DQ916502
Littorinimorpha	Hipponicidae	*Leptonetis perplexus*	AF546075
Littorinimorpha	Littorinidae	*Bembicium nanum*	FJ516230
Littorinimorpha	Littorinidae	*Echinolittorina riisei*	AJ623043
Littorinimorpha	Naticidae	*Natica vittata*	EU332642
Littorinimorpha	Naticidae	*Tectonatica sagraiana*	EU332648
Littorinimorpha	Rissoidae	*Rissoa panhormensis*	GU178011
Littorinimorpha	Vermetidae	*Dendropoma petraeum*	EU495076
Littorinimorpha	Vermetidae	*Serpulorbis* sp.	AY296830
Ptenoglossa	Cerithiopsidae	*Ataxocerithium* sp.	AY296835
Ptenoglossa	Epitoniidae	*Epitonium trevelyanum*	EF528301
Ptenoglossa	Epitoniidae	*Surrepifungium patamakanthini*	EU216021
Ptenoglossa	Eulimidae	*Balcis eburnea*	AF120636
Ptenoglossa	Eulimidae	*Thyca crystallina*	FJ386371
3B. **Pteropod Outgroup: opisthobranchs**			
Aplysiomorpha	Akeridae	*Akera bullata*	AF156143
Aplysiomorpha	Aplysiidae	*Aplysia oculifera*	AF343432
Aplysiomorpha	Aplysiidae	*Dolabella auricularia*	AF156148
Architectibranchia	Acteonidae	*Pupa strigosa*	NC002176
Architectibranchia	Acteonidae	*Acteon tornatilis*	DQ974649
Architectibranchia	Hydatinidae	*Hydatina physis*	DQ974651
Architectibranchia	Hydatinidae	*Micromelo undatus*	DQ974653
Architectibranchia	Bullinidae	*Bullina lineata*	AY296847
Cephalaspidea	**Haminoeidae**	***Atys curta***	**DQ974672**
Cephalaspidea	**Bullidae**	***Bulla vernicosa***	**DQ974662**
Cephalaspidea	Aglajidae	*Chelidonura africana*	DQ974654
Cephalaspidea	Haminoeidae	*Haminoea virescens*	AF156142
Cephalaspidea	Philinidae	*Philine scabra*	EF528306
Cephalaspidea	Runcinidae	*Runcina africana*	DQ974685
Cephalaspidea	Gastropteridae	*Sagaminopteron psychedelicum*	DQ974667
Cephalaspidea	Scaphandridae	*Scaphander lignarius*	DQ974664
Cephalaspidea	Gastropteridae	*Siphopteron tigrinum*	DQ974668
Cephalaspidea	Smaragdinellidae	*Phanerophthalmus* sp.	DQ974686
Cephalaspidea	Smaragdinellidae	*Smaragdinella* sp.	DQ974682
Cephalaspidea	Diaphanidae	*Diaphana* sp.	DQ974666
Cephalaspidea	Diaphanidae	*Toledonia globosa*	EF489395
Cephalaspidea	Retusidae	*Volvulella* sp.	DQ974684
Cephalaspidea	Retusidae	*Retusa* sp.	DQ974679
Nudipleura	**Pleurobranchidae**	***Berthella sideralis***	AJ223257
Nudipleura	Glaucidae	*Caloria indica*	DQ417325
Nudipleura	Dendronotidae	*Dendronotus frondosus*	AJ223261
Nudipleura	Flabellinidae	*Flabellina verrucosa*	AB180830
Nudipleura	Tergipedidae	*Phestilla minor*	DQ417313
Nudipleura	Polyceridae	*Plocamopherus maderae*	EF142905
Nudipleura	Zephyrinidae	*Janolus cristatus*	AF249813
Nudipleura	Arminidae	*Armina lovenii*	AF249781
Sacoglossa	Limapontiidae	*Alderia modesta*	DQ364309
Sacoglossa	**Volvatellidae**	***Ascobulla* sp.**	**DQ974683**
Sacoglossa	Boselliidae	*Bosellia mimetica*	DQ471215
Sacoglossa	Costasiellidae	*Costasiella ocellifera*	DQ471253
Sacoglossa	Placobranchidae	*Elysia tuca*	DQ781365
Sacoglossa	Polybranchiidae	*Cyerce nigricans*	DQ237995
Umbraculida	**Umbraculidae**	***Umbraculum mediterraneum***	**AY345023**
Umbraculida	Tylodinidae	*Tylodina perversa*	AF249809

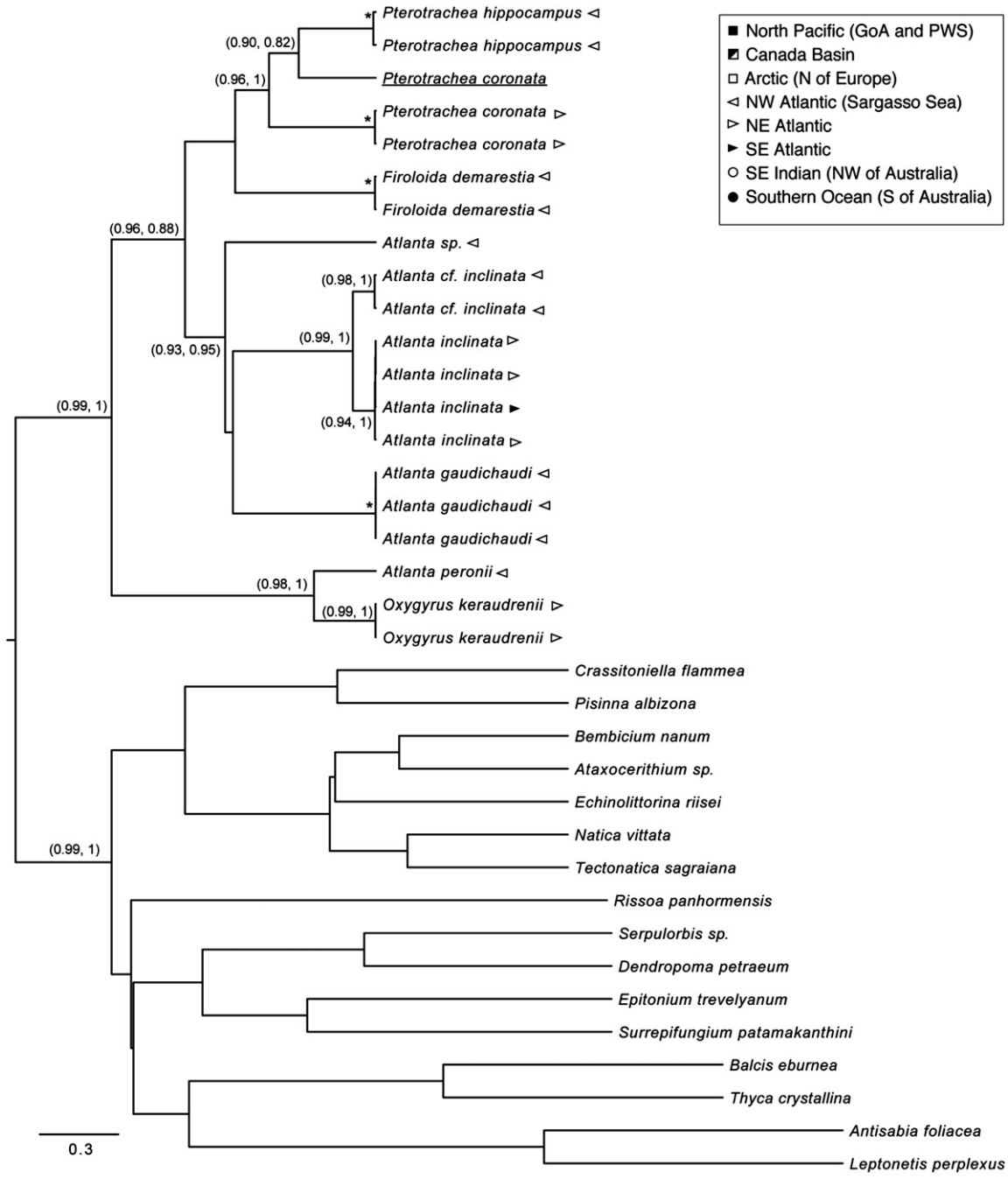

Fig. 2. Single best COI gene tree for heteropods recovered by maximum likelihood (ML), with support from ML and Bayesian analyses. Branch lengths are proportional to the amount of inferred change under ML, indicated by scale bar. Numbers in parentheses are given as (aLRT, PP); "nr" indicates topologies not recovered in the respective analysis. Asterisks indicate support of (1.00, 1.00). Underlined species denote sequences obtained from GenBank. GoA, Gulf of Alaska; PWS, Prince Williams Sound.

the appropriate burn-in for estimation of posterior probabilities (PP). Nodes were considered well supported if their aLRT and/or PP were greater than 0.90. The collection location of specimens was mapped onto the trees to investigate geographical patterns of DNA sequence variation within and among ocean basins.

3. Results

3.1. Phylogenetic analyses

Extremely similar topologies were inferred for heteropods by both ML and Bayesian methods, with high support for monophyly of Heteropoda (0.99, 1[1]; Fig. 2). Likewise, ML and Bayesian inference produced COI gene trees in which Pteropoda was highly supported as monphyletic using the all-opisthosome outgroup (1, 0.98; Supplementary Fig. 1). When ML and Bayesian trees for pteropods were inferred again with a reduced outgroup consisting of the five closest lineages (*Berthella sideralis*, *Ascobulla* sp., *Umbraculum mediterraneum*, *Atys curta*, and *Bulla vernicosa*), support for Pteropoda remained high (1, 1) while the remaining topology and support values were not substantially affected in either ML or Bayesian inference (Figs. 3 and 4).

[1] All support values in text and figures are reported in the form (aLRT, PP).

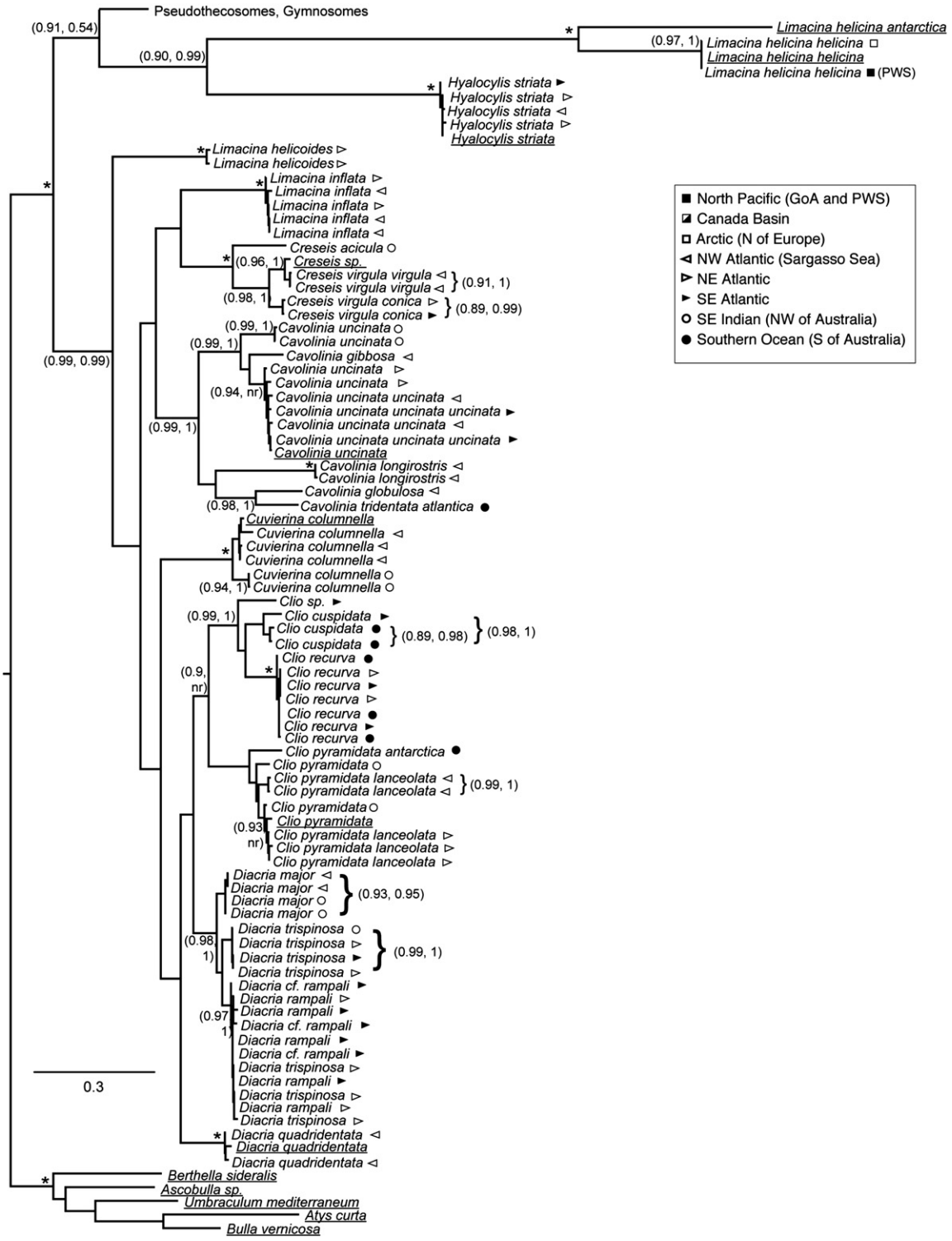

Fig. 3. Single best COI gene tree for euthecosome pteropods recovered by ML, with support from ML and Bayesian analyses. Branch lengths, nodal support, and underlined species are depicted as in Fig. 2.

In contrast to the deepest nodes, COI gene trees supported few established or proposed supra-generic taxa within Heteropoda and Pteropoda. Neither of the two families of heteropods present (Pterotracheidae and Atlantidae) was monophyletic, due to low support for Pterotracheidae, and paraphyly for the Atlantidae (Fig. 2). The Euthecosomata were not monophyletic unless *Hyalocylis striata* and *Limacina helicina* sequences were excluded (then 0.99, 0.99). Pseudothecosomata received marginal support from ML analysis and high support from Bayesian analysis (0.86, 0.99), whereas Gymnosomata received support $<50\%$ from both analyses. Gene trees also did not support a branching order regarding shelled, partially shelled, and unshelled groups in either Heteropoda or Pteropoda. Of the species for which multiple specimens were collected, monophyly was supported in the combined trees for five out of six species of heteropods (Fig. 2), and 12 out of 16 species of pteropods (Figs. 3 and 4). Barcodes from three out of four heteropod genera were monophyletic in both trees, as were barcodes from five out of seven pteropod

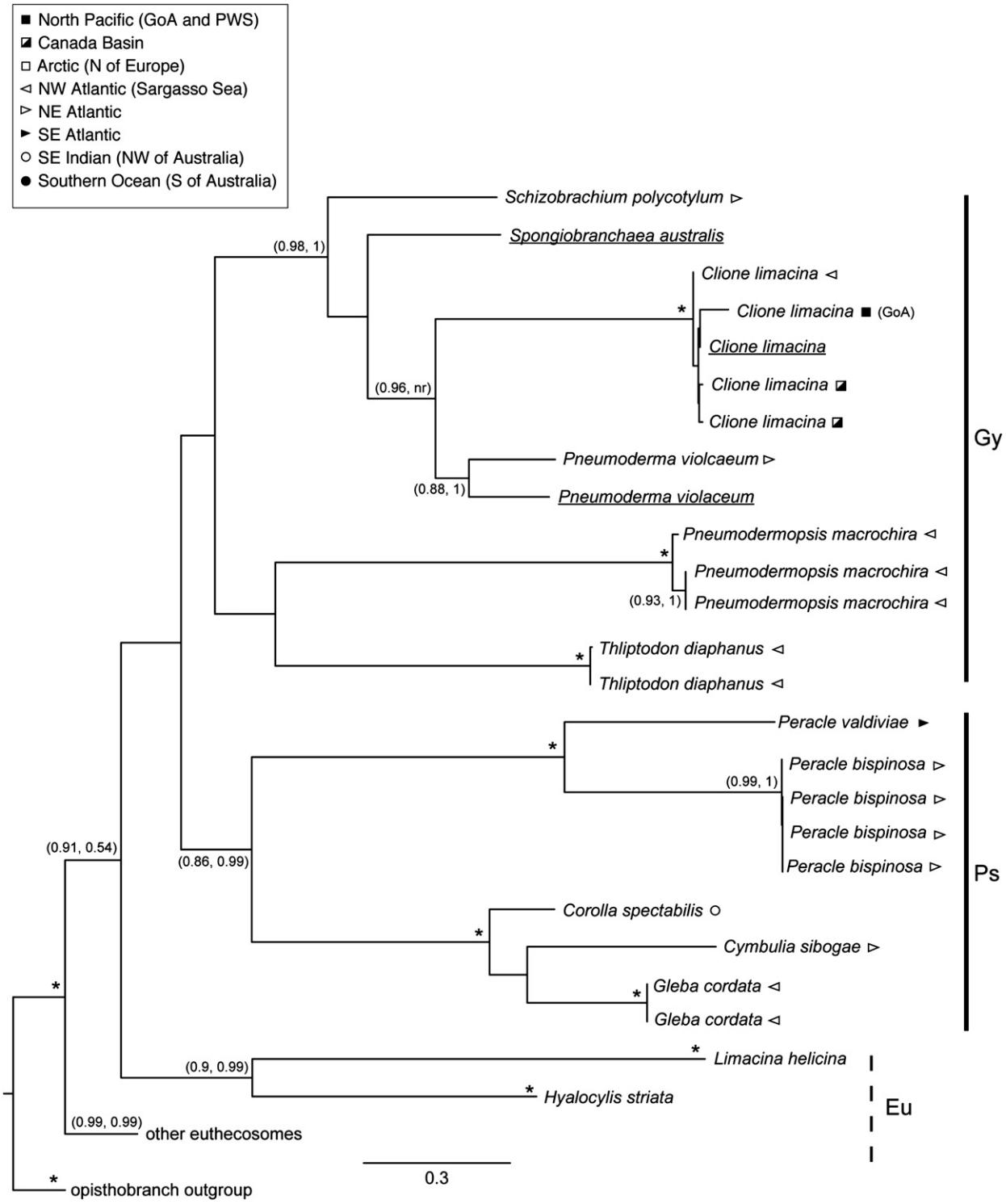

Fig. 4. Single best COI gene tree for pseudothecosome and gymnosome pteropods recovered by ML, with support from ML and Bayesian analyses. Branch lengths, nodal support, and underlined species are depicted as in Fig. 2. Gy=Gymnosomata; Ps=Pseudothecosomata; Eu=Euthecosomata.

genera. *Atlanta* and *Limacina* were not supported as monophyletic in either tree; *Clio* was supported in the ML but not the Bayesian tree, and *Diacria* by the Bayesian but not the ML tree.

3.2. Patterns of intraspecific and geographic sequence variation

Hierarchical K2P distances highlighted several features of DNA sequence variation and underscored the pattern of resolution exhibited by the gene trees (Fig. 5, Table 4). The unusually large distances between subspecies of *Limacina helicina* (0.316; marked on Fig. 5 with asterisk) were noticeable outliers; these values were excluded for computation of species level summary statistics for pteropods. Distances between the sequence of *Pterotrachea coronata* obtained from GenBank and those obtained here were similarly conspicuous (Fig. 5, marked with asterisk), probably as a result of incorrect species designation of the GenBank sequence (which was more similar to our

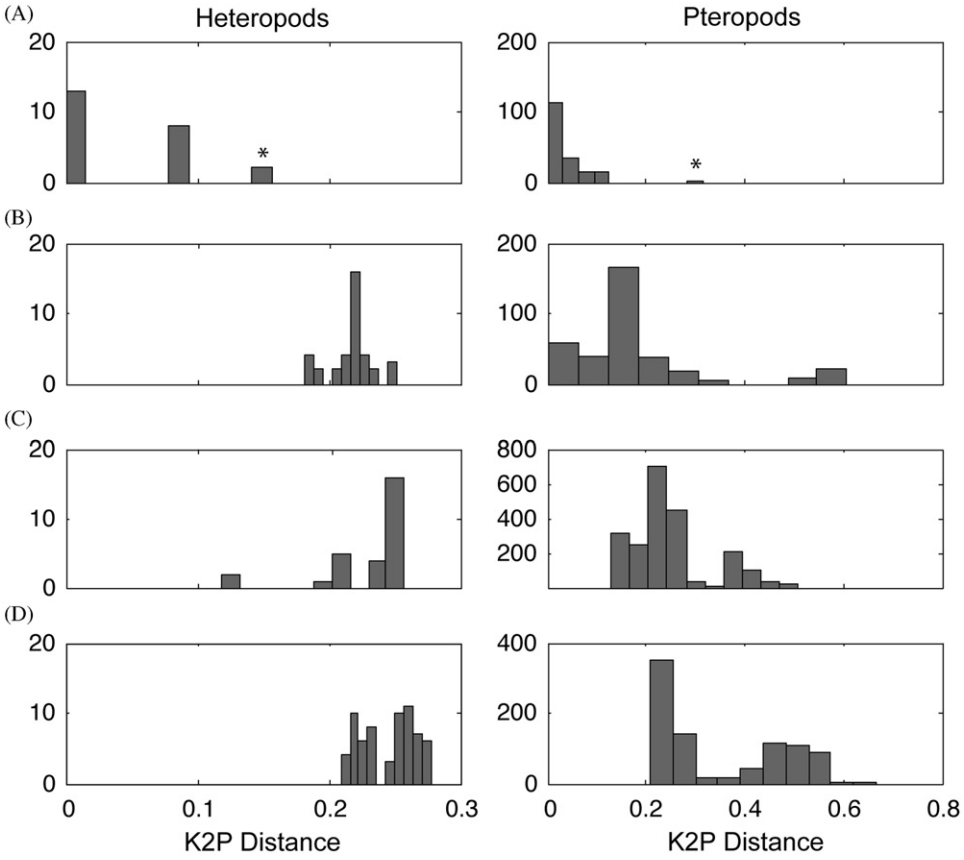

Fig. 5. Histograms of Kimura-2-parameter (K2P) genetic distance between pairs of COI sequences in this study, separately for heteropods and pteropods at four hierarchical taxonomic levels: A, within each species, B, among species within each genus, C, among genera within each family, and D, among families within each major taxonomic group (e.g. euthecosomes, gymnosomes, etc.). Asterisks mark aberrant values that were removed from the data before statistical summary.

Table 4
Descriptive statistics for pairwise K2P distances depicted in the histograms of Fig. 5. N, number of comparisons; SD, standard deviation; min, minimum value; max, maximum value.

Taxonomic Level	Heteropods				Pteropods			
	N	Mean	SD	[min, max]	N	Mean	SD	[min, max]
Within Species	21	0.0328	0.0399	[0, 0.0859]	177	0.0302	0.0334	[0, 0.117]
Between Species, Within each Genus	37	0.217	0.0172	[0.181, 0.252]	356	0.176	0.131	[0.0015, 0.608]
Between Genera, Within each Family	28	0.230	0.0354	[0.119, 0.258]	2112	0.247	0.0778	[0.129, 0.509]
Between Major Groups	65	0.245	0.0206	[0.209,0.278]	898	0.351	0.125	[0.208, 0.667]
Total	153				3543			

P. hippocampus). These numbers were also excluded from species level summary statistics for heteropods. Pairwise K2P mean distances and standard deviations increased at higher taxonomic levels, but demonstrated a clearer "barcode gap" for heteropods than for pteropods. The two highest taxonomic levels showed evidence of multimodality (Fig. 5). Note that, although the standard deviations give an indication of the dispersion of K2P distances, distributional tests based on mean and variance are not valid because pairwise comparisons are not independent.

Although sample sizes per species per locality for holoplanktonic gastropods were small, the barcodes did indicate some potential geographic patterns in intraspecific COI sequence variation that warrant further exploration. No substantial regional variation was detected among Atlantic locations for *Cavolinia uncinata*, *Creseis virgula conica*, *Hyalocylis striata*, or *Limacina inflata*. Conspecific specimens from widely separated ocean basins were genetically similar in several cases: *Clio recurva* (E Atlantic and Southern Ocean), *Clione limacina* (NW Atlantic, Canada Basin, and Gulf of Alaska), *Diacria trispinosa* (E Atlantic and SE Indian), and *Limacina helicina helicina* (Prince Williams Sound and Arctic). Conversely, sequence differences were detected between regions in *Cavolinia uncinata* (Atlantic vs. SE Indian), *Clio cuspidata* (SE Atlantic vs. Southern Ocean), *Cuvierina columnella* (NW Atlantic vs. SE Indian), and *Diacria major* (NW Atlantic vs. SE Indian).

4. Discussion

Distributions of pairwise K2P distances between individuals were consistent with values reported in COI for other taxa (Fig. 5, Table 4). The mean distance between individuals within a species was 0.0328 for heteropods and 0.0302 for pteropods. These values are mostly higher than intraspecific K2P means reported in recent barcoding work: 0.00460 (decapods, Costa et al., 2007), 0.00740

(gammarid amphipods, Costa et al., 2007), and 0.0145 (chaetognaths, Jennings et al., 2010). Bucklin et al. (2010) reported intraspecific means of 0.00130-0.0388 across ten broad taxonomic groups; Meier et al. (2008) calculated uncorrected sequence divergences of 0.026 for gastropods, and 0.007-0.050 for other invertebrate groups, though these are not directly comparable to K2P distances. The mean pairwise distance between species within each genus was 0.217 for heteropods and 0.176 for pteropods, more within the range of values in the studies cited above: 0.170 (decapods), 0.250 (amphipods), 0.345 (chaetognaths), and 0.185-0.477 (Bucklin et al., 2010). The uncorrected mean divergences of Meier et al. (2008) were 0.112 (gastropods), and 0.062-0.158 (other invertebrates). It is important to note firstly that our pairwise values (and those of Jennings et al., 2010) were computed as in Meier et al. (2008)—that is, we computed interspecific distances only between congeneric species pairs, whereas the work cited above computed distances between all interspecific pairs. Secondly, presenting histograms in addition to mean $\pm$ SD allows a more nuanced reading of the distance structure. Although only heteropod histograms showed an idealized "barcode gap" (no distributional overlap at the lowest two levels), in most cases barcodes of both pteropods and heteropods did group strongly by species in the trees, indicating that they are still useful as an aid in species designation. This situation highlights the difference between mean vs. minimum distances, (Goetze, 2010; Meier et al., 2008), but also the overreliance on distance methods in general: more of the information contained in DNA sequences is used in Bayesian and ML tree-building algorithms than in the reduction to distance matrices, resulting in greater resolution. The large range of pairwise distances in pteropods and heteropods appears to mirror the high morphological diversity of these taxa. Although the histograms' dependence on correctly specified taxonomy complicates their use in poorly understood groups where such may be uncertain, the exploratory power of barcodes to draw attention to these neglected groups can help frame appropriate questions and direct future work. In this case, it seems apparent that the philosophical dichotomy between "lumping" and "splitting" species and forms has affected the taxonomy of both groups, but that barcodes can aid in assessing and revising these divisions.

In most species for which multiple subspecies or forms were collected, the forms were genetically distinct. The sequences for *Limacina helicina helicina* were strongly differentiated from *L. helicina antarctica*, and with K2P distances (0.316) more typical of separate pteropod species. *Creseis virgula conica* and *C. virgula virgula* were also separated genetically, although the pattern was complicated by the lack of full species identification for the intervening *Creseis* sp. sequence obtained from GenBank. Although the determination for *Clio pyramidata lanceolata* vs. *C. pyramidata antarctica* was also unclear due to incompletely identifiable specimens, the fully identified specimens pointed towards genetic separation of the forms. Preliminary analysis of sequences from *Clio pyramidata excisa* (Hopcroft unpublished data) indicate that both *excisa* and *lanceolata* are genetically differentiated forms. In contrast, genetic distinctness was not clearly supported for *Cavolinia uncinata uncinata*. On the whole, genetic divergence among the differentiated forms appeared more typical of divergence between full species.

It is interesting to note that the cases of regional sequence differences within species involved specimens from the southeastern Indian Ocean (*Cavolinia uncinata* and *Cuvierina columnella*) or Southern Ocean (*Clio cuspidata*). If these sequences do not represent separate forms or subspecies, this separation would alternatively imply that restricted gene flow between regions may sometimes play a role in shaping patterns of molecular variation in holoplanktonic snails. Further material and analysis (both morphological and molecular) will be needed to fully determine the status of all of these cases.

Barcode analysis was also useful in discriminating species of gymnosomes and pseudothecosomes, although taxonomic sampling was much sparser than of euthecosomes (Fig. 4). Only in the genus *Peracle* was more than one species barcoded; yet, the close genetic association of multiple specimens of *Gleba cordata*, *Thliptodon diaphanous*, *Clione limacina*, and *Pneumodermopsis macrochira* indicates that barcodes are potentially useful in these taxa. Of particular interest would be barcodes of Southern Ocean *Clione limacina*, which has been described alternatively as a subspecies (*C. limacina antarctica*) and a separate species (*Clione antarctica*; see Gilmer and Lalli, 1990); emerging work indicates that the two are not genetically similar enough to represent a single bipolar species (Allcock et al., unpublished).

Several genera of holoplanktonic gastropods displayed unexpected patterns of inter- and intraspecific diversity, which could reflect inaccurate taxonomic status, misidentification, cryptic variation, or lack of resolution in COI. Among heteropods, the distinctness of the two *Atlanta cf. inclinata* specimens (which had a golden color to the keel on the shell) from *A. inclinata* (which possesses clear keels) raises the possibility of cryptic speciation. The sequence for *Atlanta peronii* prevented *Atlanta* from being a monophyletic genus. In the keys of Tesch (1949) and van der Spoel (1976), the distinguishing features between *Atlanta* and *Oxygyrus* are a matter of degree rather than presence/absence (e.g. degree of planorbicity of shell, nature and extent of keel), and moreover these "characters" are similar to the distinctions between species of *Atlanta*. Species of *Atlanta* were so difficult to assess morphologically that they were placed into tentative species "groups" or "complexes" by these authors, with the observation of frequent gradations in between forms. More sampling effort needs to be focused on these extremely neglected heteropods, so that both morphological and molecular reanalysis can be used to improve our understanding of their taxonomy.

Similarly to heteropods, within pteropods the barcodes for *Limacina* species showed exceptional genetic divergences; although all three species were monophyletic individually, they appeared on separate branches in the COI trees (Fig. 3). Note that van der Spoel (1976) placed *L. helicina* and *L. retroversa* in the subgenus *Limacina*, whereas *L. helicoides*, *L. inflata*, and *L. lesueuri* were placed in the subgenus *Thilea*, and *L. bulimoides*, *L. trochiformis*, and *L. cochlostyloides* in the subgenus *Munthea*. Remigio and Hebert (2003) also reported long branches and divergent relationships for *Limacina helicina* compared to other opisthosomes, and other independently obtained COI sequences of euthecosomes have produced this external placement of *Limacina helicina* (K. Peijnenburg, unpublished data; J. Strugnell et al., unpublished data) and *Hyalocylis striata* (K. Peijnenburg, unpublished data). Although these repeated and independent findings of similar relationships are encouraging, at present time there seems to be no obvious explanation for the apparently divergent lineages of *Limacina*.

Barcodes could not aid or confirm morphological determinations in two other cases. The potentially undescribed species *Clio* sp., which was collected from the deep Angola Basin and was white in color (unusual for *Clio*), did not match any barcoded species in our collection or available in GenBank. Closer morphological examination revealed similarities to the rare *Clio orthotheca* from the Bay of Bengal, but neither morphological nor genetic analysis could provide a complete identity for this specimen. Also, sequences of *Diacria trispinosa*, *D. rampali*, and *D. cf. rampali* were highly mixed, while *D. major* and *D. quadridentata* were monophyletic. Four grouped sequences of *D. trispinosa* likely represent the true species branch. However, all specimens of the three mixed species were identified by the same

taxonomist and the differences between the species appeared consistent between several stations, raising the possibility of a gradient of morphology whose genetic underpinning is unclear. It is possible that these lineages have separated too recently to show reciprocal monophyly in COI, but further barcoding and careful morphological evaluation are needed to address this issue.

Finally, high phylogenetic support for a monophyletic Pteropoda and a monophyletic Heteropoda strongly implies that both groups represent single radiations into the pelagic realm. Monophyly of each group was recovered even with greatly expanded outgroup sequences from diverse gastropod groups, and expanded ingroup sequences as compared to previous work (Klussmann-Kolb and Dinapoli, 2006; Remigio and Hebert, 2003). The lack of resolution typical of COI at intermediate nodes (Schander and Willassen, 2005; Stoeckle and Hebert, 2008) precluded conclusive determination of the evolutionary history within either group. However, the molecular analysis of Klussmann-Kolb and Dinapoli (2006) combined COI with the nuclear large and small subunit rDNAs (18S and 28S), and indicated the potential for nuclear ribosomal sequences from the larger taxonomic sampling in this study to provide better resolution to intermediate nodes. Such an expanded multi-gene approach may allow testing of longstanding evolutionary hypotheses concerning these two gastropod lineages (e.g. monophyly of shell types of heteropods and pteropods, and progressive loss of shell with further adaptation to the pelagic realm).

5. Conclusions

Barcoding of pteropod and heteropod specimens from the global ocean revealed complex patterns of COI sequence variation that could be a result of their life histories and evolution in the pelagic realm. While the forms of most species exhibited large genetic differences, monophyly of some forms could not be confirmed. For most species for which no subspecific divisions were previously known, barcoding revealed higher than average sequence variation in lineages from different oceans, especially the southeast Indian and Southern Oceans, and even from different regions of the same ocean. Given that several described forms do appear to be genetically distinct, it is unclear whether the regional differences of these specimens represent novel forms, described forms that could not be fully identified in this work, or truly cryptic species. Barcodes were not universally effective in sorting sequences from congeneric or occasionally even confamilial species; while these mixed results highlight the often subtle morphological differences inherent in current holoplanktonic gastropod taxonomies, they may also reflect the inadequacy of summary procedures such as determination of a "barcode gap" or reporting only of means and standard deviations of inter- vs. intraspecific distances. Analysis of the barcodes in this work provides the first molecular data highlighting the need for taxonomic revision, and underscores the need to increase both taxonomic coverage and geographic sampling. Although a true phylogeographic study would require immense sampling efforts even for a few species, the preliminary patterns of regional sequence variation at present indicate that such an analysis would be very interesting, particularly between species exhibiting different life history traits (such as mobility and reproductive mode).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dsr2.2010.09.022.

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